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<b>(54) Title:</b> EXPRESSION OF G PROTEIN COUPLED RECEPTORS IN YEAST  <b>(57) Abstract</b> <p>Disclosed is a transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G protein <math>\alpha</math> subunit (mammalian <math>G_\alpha</math>). The first and second heterologous DNA sequences are capable of expression in the cell, but the cell is incapable of expressing an endogenous G protein <math>\alpha</math>-subunit (yeast <math>G_\alpha</math>). The cells are useful for screening compounds which affect the rate of dissociation of <math>G_\alpha</math> from <math>G_{\beta\gamma}</math> in a cell. Also disclosed is a novel DNA expression vector useful for making cells as described above. The vector contains a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor. A second segment is positioned downstream from the first segment (and in correct reading frame therewith), with the second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor.</p>		

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## EXPRESSION OF G PROTEIN COUPLED RECEPTORS IN YEAST

This invention was made with government support under NIH grant HL16037. The government may have certain rights to this invention.

### Field of the Invention

5           This invention relates to yeast cells expressing heterologous G protein coupled receptors, vectors useful for making such cells, and methods of using the same.

### Background of the Invention

10           The actions of many extracellular signals (for example, neurotransmitters, hormones, odorants, light) are mediated by receptors with seven transmembrane domains (G protein coupled receptors) and heterotrimeric guanine nucleotide-binding regulatory  
15           proteins (G proteins). See H. Dohlman, M. Caron, and R. Lefkowitz, Biochemistry **26**, 2657 (1987); L. Stryer and H. Bourne, Ann. Rev. Cell Biol. **2**, 391 (1986). Such G protein-mediated signaling systems have been identified in organisms as divergent as yeast and man. See H.  
20           Dohlman et al., supra; L. Stryer and H. Bourne, supra; K. Blumer and J. Thorner, Annu. Rev. Physiol. (in press). The  $\beta$ 2-adrenergic receptor ( $\beta$ AR) is the prototype of the seven-transmembrane-segment class of

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ligand binding receptors in mammalian cells. In response to epinephrine or norepinephrine,  $\beta$ AR activates a G protein,  $G_s$ , which in turn stimulates adenylyate cyclase and cyclic adenosine monophosphate production in the cell. See H. Dohlman et al., supra;  
5 L. Stryer and H. Bourne, supra. G protein-coupled pheromone receptors in yeast control a developmental program that culminates in mating (fusion) of  $a$  and  $\alpha$  haploid cell types to form the  $a/\alpha$  diploid. See K.  
10 Blumer and J. Thorner, supra; I. Herskowitz, Microbiol. Rev. 52, 536 (1988).

The present invention is based on our continued research into the expression of heterologous G protein coupled receptors in yeast.

15 Summary of the Invention

A first aspect of the present invention is a transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence  
20 which codes for a mammalian G protein  $\alpha$  subunit (mammalian  $G_\alpha$ ). The first and second heterologous DNA sequences are capable of expression in the cell, but the cell is incapable of expressing an endogenous G protein  $\alpha$ -subunit (yeast  $G_\alpha$ ). The cell optionally  
25 contains a third heterologous DNA sequence, with the third heterologous DNA sequence comprising a pheromone-responsive promotor and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith.

30 A second aspect of the present invention is a method of testing a compound for the ability to affect the rate of dissociation of  $G_\alpha$  from  $G_{\beta\gamma}$  in a cell. The method comprises: providing a transformed yeast cell as described above; contacting the compound to the cell;  
35 and then detecting the rate of dissociation of  $G_\alpha$  from  $G_{\beta\gamma}$  in the cell. The cells may be provided in an

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aqueous solution, and the contacting step carried out by adding the compound to the aqueous solution.

A third aspect of the present invention is a DNA expression vector capable of expressing a transmembrane protein into the cell membrane of yeast cells. The vector contains a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor. A second segment is positioned downstream from the first segment (and in correct reading frame therewith), with the second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor.

A fourth aspect of the present invention is a yeast cell transformed by a vector as described above.

#### Brief Description of the Drawings

Figure 1 illustrates the construction of the yeast human  $\beta 2$  Adrenergic Receptor expression plasmid, pY $\beta$ AR2.

Figure 2 illustrates h $\beta$ AR ligand binding to membranes from pY $\beta$ AR2-transformed yeast cells.

Figure 3 shows a comparison of  $\beta$ -adrenergic agonist effects on pheromone-inducible gene activity.  $\alpha$ -MF, 10  $\mu$ M  $\alpha$ -mating factor; (-) ISO, 50  $\mu$ M (-) isoproterenol; (-) ALP, 50  $\mu$ M (-) alprenolol; (+) ISO, 100  $\mu$ M (+) isoproterenol.

#### Detailed Description of the Invention

Nucleotide bases are abbreviated herein as follows:

A=Adenine	G=Guanine
C=Cytosine	T=Thymine

Amino acid residues are abbreviated herein to either three letters or a single letter as follows:

Ala;A=Alanine	Leu;L=Leucine
Arg;R=Arginine	Lys;K=Lysine
Asn;N=Asparagine	Met;M=Methionine
Asp;D=Aspartic acid	Phe;F=Phenylalanine
Cys;C=Cysteine	Pro;P=Proline

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Gln;Q=Glutamine	Ser;S=Serine
Glu;E=Glutamic acid	Thr;T=Threonine
Gly;G=Glycine	Trp;W=Tryptophan
His;H=Histidine	Tyr;Y=Tyrosine
Ile;I=Isoleucine	Val;V=Valine

5

The term "mammalian" as used herein refers to any mammalian species (e.g., human, mouse, rat, and monkey).

10 The term "heterologous" is used herein with respect to yeast, and hence refers to DNA sequences, proteins, and other materials originating from organisms other than yeast (e.g., mammalian, avian, amphibian), or combinations thereof not naturally found in yeast.

15 The terms "upstream" and "downstream" are used herein to refer to the direction of transcription and translation, with a sequence being transcribed or translated prior to another sequence being referred to as "upstream" of the latter.

20 G proteins are comprised of three subunits: a guanyl-nucleotide binding  $\alpha$  subunit; a  $\beta$  subunit; and a  $\gamma$  subunit. G proteins cycle between two forms, depending on whether GDP or GTP is bound thereto. When GDP is bound the G protein exists as an inactive heterotrimer, the  $G_{\alpha\beta\gamma}$  complex. When GTP is bound the  $\alpha$  subunit dissociates, leaving a  $G_{\beta\gamma}$  complex. Importantly, when a  $G_{\alpha\beta\gamma}$  complex operatively associates with an activated G protein coupled receptor in a cell membrane, the rate of exchange of GTP for bound GDP is increased and, hence, the rate of dissociation of the bound  $\alpha$  subunit from the  $G_{\beta\gamma}$  complex increases. This fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena. See generally Stryer and Bourne, supra.

35 Any mammalian G protein coupled receptor, and the DNA sequences encoding these receptors, may be employed in practicing the present invention. Examples

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of such receptors include, but are not limited to, dopamine receptors, muscarinic cholinergic receptors,  $\alpha$ -adrenergic receptors,  $\beta$ -adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin  
5 receptors. The term receptor as used herein is intended to encompass subtypes of the named receptors, and mutants and homologs thereof, along with the DNA sequences encoding the same.

The human D<sub>1</sub> dopamine receptor cDNA is  
10 reported in A. Dearry et al., Nature **347**, 72-76 (1990).

The rat D<sub>2</sub> dopamine receptor cDNA is reported in J. Bunzow et al., Nature **336**, 783-787 (1988); see also O. Civelli, et al., PCT Appln. WO 90/05780 (all  
15 references cited herein are to be incorporated herein by reference).

Muscarinic cholinergic receptors (various subtypes) are disclosed in E. Peralta et al., Nature **343**, 434 (1988) and K. Fukuda et al., Nature **327**, 623  
20 (1987).

Various subtypes of  $\alpha_2$ -adrenergic receptors are disclosed in J. Regan et al., Proc. Natl. Acad. Sci. USA **85**, 6301 (1988) and in R. Lefkowitz and M. Caron, J. Biol. Chem. **263**, 4993 (1988).

Serotonin receptors (various subtypes) are  
25 disclosed in S. Peroutka, Ann. Rev. Neurosci. **11**, 45 (1988).

A cannabinoid receptor is disclosed in L. Matsuda et al., Nature **346**, 561 (1990).

Any DNA sequence which codes for a mammalian  
30 G  $\alpha$  subunit (G $\alpha$ ) may be used to practice the present invention. Examples of mammalian G  $\alpha$  subunits include G<sub>s</sub>  $\alpha$  subunits, G<sub>i</sub>  $\alpha$  subunits, G<sub>o</sub>  $\alpha$  subunits, G<sub>z</sub>  $\alpha$  subunits, and transducin  $\alpha$  subunits. See generally Stryer and Bourne, supra. G proteins and subunits  
35 useful for practicing the present invention include subtypes, and mutants and homologs thereof, along with the DNA sequences encoding the same.

vector is a replicable DNA construct in which a DNA  
sequence encoding the heterologous DNA sequence is  
5 operably linked to suitable control sequences capable  
of effecting the expression of a protein or protein  
subunit coded for by the heterologous DNA sequence in  
the intended host. Generally, control sequences  
include a transcriptional promoter, an optional  
10 operator sequence to control transcription, a sequence  
encoding suitable mRNA ribosomal binding sites, and  
(optionally) sequences which control the termination of  
transcription and translation.

Vectors useful for practicing the present  
15 invention include plasmids, viruses (including phage),  
and integratable DNA fragments (i.e., fragments  
integratable into the host genome by homologous  
recombination). The vector may replicate and function  
independently of the host genome, as in the case of a  
20 plasmid, or may integrate into the genome itself, as in  
the case of an integratable DNA fragment. Suitable  
vectors will contain replicon and control sequences  
which are derived from species compatible with the  
intended expression host. For example, a promoter  
25 operable in a host cell is one which binds the RNA  
polymerase of that cell, and a ribosomal binding site  
operable in a host cell is one which binds the  
endogenous ribosomes of that cell.

DNA regions are operably associated when they  
30 are functionally related to each other. For example: a  
promoter is operably linked to a coding sequence if it  
controls the transcription of the sequence; a ribosome  
binding site is operably linked to a coding sequence if  
it is positioned so as to permit translation.  
35 Generally, operably linked means contiguous and, in the  
case of leader sequences, contiguous and in reading  
phase.



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Transformed host cells of the present invention are cells which have been transformed or transfected with the vectors constructed using recombinant DNA techniques and express the protein or protein subunit coded for by the heterologous DNA sequences. In general, the host cells are incapable of expressing an endogenous G protein  $\alpha$ -subunit (yeast  $G_{\alpha}$ ). The host cells do, however, express a complex of the G protein  $\beta$  subunit and the G protein  $\gamma$  subunit ( $G_{\beta\gamma}$ ). The host cells may express endogenous  $G_{\beta\gamma}$ , or may optionally be engineered to express heterologous  $G_{\beta\gamma}$  (e.g., mammalian) in the same manner as they are engineered to express heterologous  $G_{\alpha}$ .

A variety of yeast cultures, and suitable expression vectors for transforming yeast cells, are known. See, e.g., U.S. Patent No. 4,745,057; U.S. Patent No. 4,797,359; U.S. Patent No. 4,615,974; U.S. Patent No. 4,880,734; U.S. Patent No. 4,711,844; and U.S. Patent No. 4,865,989. Saccharomyces cerevisiae is the most commonly used among the yeast, although a number of other strains are commonly available. See, e.g., U.S. Patent No. 4,806,472 (Kluveromyces lactis and expression vectors therefor); 4,855,231 (Pichia pastoris and expression vectors therefor). Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding the heterologous DNA sequences, sequences for polyadenylation and transcription termination, and a selection gene. An exemplary plasmid is YRp7, (Stinchcomb et al., Nature 282, 39 (1979); Kingsman et al., Gene 7, 141 (1979); Tschemper et al., Gene 10, 157 (1980)). This plasmid contains the TRP1 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, Genetics 85, 12 (1977)). The presence of the TRP1 lesion in the

yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors  
5 include the promoters for metallothionein,  
3-phosphoglycerate kinase (Hitzeman et al., J. Biol.  
Chem. 255, 2073 (1980) or other glycolytic enzymes  
(Hess et al., J. Adv. Enzyme Reg. 7, 149 (1968); and  
Holland et al., Biochemistry 17, 4900 (1978)), such as  
10 enolase, glyceraldehyde-3-phosphate dehydrogenase,  
hexokinase, pyruvate decarboxylase,  
phosphofructokinase, glucose-6-phosphate isomerase,  
3-phosphoglycerate mutase, pyruvate kinase,  
triosephosphate isomerase, phosphoglucose isomerase,  
15 and glucokinase. Suitable vectors and promoters for  
use in yeast expression are further described in R.  
Hitzeman et al., EPO Publn. No. 73,657. Other  
promoters, which have the additional advantage of  
transcription controlled by growth conditions, are the  
20 promoter regions for alcohol dehydrogenase 2,  
isocytochrome C, acid phosphatase, degradative enzymes  
associated with nitrogen metabolism, and the  
aforementioned metallothionein and  
glyceraldehyde-3-phosphate dehydrogenase, as well as  
25 enzymes responsible for maltose and galactose  
utilization.

In constructing suitable expression plasmids,  
the termination sequences associated with these genes  
may also be ligated into the expression vector 3' of  
30 the heterologous coding sequences to provide  
polyadenylation and termination of the mRNA.

A novel DNA expression vector described  
herein which is particularly useful for carrying out  
the present invention contains a first segment  
35 comprising at least a fragment of the extreme amino-  
terminal coding sequence of a yeast G protein coupled  
receptor and a second segment downstream from said

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first segment and in correct reading frame therewith, the second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor (e.g., a mammalian G protein coupled receptor). In a preferred embodiment, this vector comprises a plasmid. In constructing such a vector, a fragment of the extreme amino-terminal coding sequence of the heterologous G protein coupled receptor may be deleted. The first and second segments are operatively associated with a promoter, such as the GAL1 promoter, which is operative in a yeast cell. Coding sequences for yeast G protein coupled receptors which may be used in constructing such vectors are exemplified by the gene sequences encoding yeast pheromone receptors (e.g., the STE2 gene, which encodes the  $\alpha$ -factor receptor, and the STE3 gene, which encodes the  $\alpha$ -factor receptor). The levels of expression obtained from these novel vectors are enhanced if at least a fragment of the 5'-untranslated region of a yeast G protein coupled receptor gene (e.g., a yeast pheromone receptor gene; see above) is positioned upstream from the first segment and operatively associated therewith.

Any of a variety of means for detecting the dissociation of  $G_\alpha$  from  $G_{\beta\gamma}$  can be used in connection with the present invention. The cells could be disrupted and the proportion of these subunits and complexes determined physically (i.e., by chromatography). The cells could be disrupted and the quantity of  $G_\alpha$  present assayed directly by assaying for the enzymatic activity possessed by  $G_\alpha$  in isolation (i.e., the ability to hydrolyze GTP to GDP). Since whether GTP or GDP is bound to the G protein depends on whether the G protein exists as a  $G_{\beta\gamma}$  or  $G_{\alpha\beta\gamma}$  complex, dissociation can be probed with radiolabelled GTP. As explained below, morphological changes in the cells can be observed. A particularly convenient method, however, is to provide in the cell a third heterologous

sequence comprises a pheromone-responsive promoter and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith. This sequence can be inserted with a vector, as described in detail herein. With such a sequence in place, the detecting step can be carried out by monitoring the expression of the indicator gene in the cell. Any of a variety of pheromone responsive promoters could be used, examples being the BAR1 gene promoter and the FUS1 gene promoter. Likewise, any of a broad variety of indicator genes could be used, with examples including the HIS3 gene and the LacZ gene.

As noted above, transformed host cells of the present invention express the protein or protein subunit coded for by the heterologous DNA sequence. When expressed, the G protein coupled receptor is located in the host cell membrane (i.e., physically positioned therein in proper orientation for both the stereospecific binding of ligands on the extracellular side of the cell membrane and for functional interaction with G proteins on the cytoplasmic side of the cell membrane).

The ability to control the yeast pheromone response pathway by expression of a heterologous adrenergic receptor and its cognate G protein  $\alpha$ -subunit has the potential to facilitate structural and functional characterization of mammalian G protein-coupled receptors. By scoring for responses such as growth arrest or  $\beta$ -galactosidase induction, the functional properties of mutant receptors can now be rapidly tested. Similarly, as additional genes for putative G protein-coupled receptors are isolated, numerous ligands can be screened to identify those with activity toward previously unidentified receptors. See F. Libert et al., Science 244, 569 (1989); M. S. Chee

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et al., Nature 344, 774 (1990). Moreover, as additional genes coding for putative G protein  $\alpha$ -subunits are isolated, they can be expressed in cells of the present invention and screened with a variety of G protein coupled receptors and ligands to characterize these subunits. These cells can also be used to screen for compounds which affect receptor-G protein interactions.

Cells of the present invention can be deposited in the wells of microtiter plates in known, predetermined quantities to provide standardized kits useful for screening compounds in accordance with the various screening procedures described above.

The following Examples are provided to further illustrate various aspects of the present invention. They are not to be construed as limiting the invention.

### EXAMPLE 1

#### Construction of the Human $\beta$ 2-Adrenergic Expression Vector pY $\beta$ AR2 and Expression in Yeast

To attain high level expression of the human  $\beta$ 2-adrenergic receptor (h $\beta$ AR) in yeast, a modified h $\beta$ AR gene was placed under the control of the GAL1 promoter in the multicopy vector, YEp24 (pY $\beta$ AR2).

Figure 1 illustrates the construction of yeast expression plasmid pY $\beta$ AR2. In pY $\beta$ AR2, expression of the h $\beta$ AR sequence is under the control of the GAL1 promoter. Figure 1A shows the 5'-untranslated region and the first 63 basepairs (bp) of coding sequence of the h $\beta$ AR gene in pTZNAR, B. O'Dowd et al., J. biol. Chem. 263, 15985 (1988), which was removed by Aat II cleavage and replaced with a synthetic oligonucleotide corresponding to 11 bp of noncoding and 42 bp of coding sequence from the STE2 gene (SEQ ID NO:1; SEQ ID NO:2). See N. Nakayama et al., EMBO J. 4, 2643 (1985); A. Burkholder and L. Hartwell, Nucleic Acids Res. 13, 8463

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(1985). The resulting plasmid, pTZYNAR, contains the modified h $\beta$ AR gene flanked by Hind III sites in noncoding sequences with the 3' Hind III site given as SEQ ID NO:3 herein. The Hind III-Hind III fragment was isolated from pTZYNAR and inserted into pAAH5 such that the 3'- untranslated sequence of the modified h $\beta$ AR gene was followed by 450 bp containing termination sequences from the yeast ADH1 gene. See G. Ammerer, Methods. Enzymol. 101, 192 (1983).

As illustrated in Figure 1B, py $\beta$ AR2 was constructed by inserting the Bam HI - Bam HI fragment containing h $\beta$ AR and ADH1 sequences into YEpG24. E. Wyckoff and T. Hsieh, Proc. Natl. Acad. Sci. U.S.A. 85, 6272 (1988). Where maximum expression was sought, cells were cotransformed with plasmid pMTL9 (from Dr. S. Johnston) containing LAC9, a homolog of the S. cerevisiae GAL4 transactivator protein required for GAL1-regulated transcription. J. Salmeron et al., Mol. Cell. Biol. 9, 2950 (1989). Cells grown to late exponential phase were induced in medium containing 3% galactose, supplemented with about 10  $\mu$ M alprenolol, and grown for an additional 36 hours. Standard methods for the maintenance of cells were used. See F. Sherman et al., Methods in Yeast Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986).

Maximal expression required (i) expression of a transcriptional transactivator protein (LAC9), (ii) replacement of the 5' untranslated and extreme NH<sub>2</sub>-terminal coding sequence of the h $\beta$ AR gene with the corresponding region of the yeast STE2 ( $\alpha$ -factor receptor) gene, (iii) induction with galactose when cell growth reached late exponential phase, and, (iv) inclusion of an adrenergic ligand in the growth medium during induction.

The plasmid py $\beta$ AR2 was deposited in accordance with the provisions of the Budapest Treaty at the American Type Culture Collection, 12301 Parklawn

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Drive, Rockville, MD 20852 USA, on September 11, 1990, and has been assigned ATCC Accession No. 40891.

## EXAMPLE 2

### Binding Affinity of h $\beta$ AR Ligands in Yeast

#### Transformed with pY $\beta$ AR2

5 A primary function of cell surface receptors is to recognize only appropriate ligands among other extracellular stimuli. Accordingly, ligand binding affinities were determined to establish the functional  
10 integrity of h $\beta$ AR expressed in yeast. As discussed in detail below, an antagonist,  $^{125}\text{I}$ -labeled cyanopindolol ( $^{125}\text{I}$ -CYP), bound in a saturable manner and with high affinity to membranes prepared from pY $\beta$ AR2-transformed yeast cells. By displacement of  $^{125}\text{I}$ -CYP with a series  
15 of agonists, the order of potency and stereospecificity expected for h $\beta$ AR was observed.

SC261 cells (MATA ura3-52 trp1 leu2 prb1-1122 pep4-3 prc1-407) (from Dr. S. Johnston) harboring pY $\beta$ AR2 (URA3) and pMTL9 (LEU2) were grown in minimal  
20 glucose-free selective media to late log phase ( $\text{OD}_{600} = 5.0$ ), and then induced with the addition of 3% galactose and 40  $\mu\text{M}$  alprenolol. After 36 hours, cells were harvested and spheroplasts were prepared as described. See E. Wyckoff and T. Hsieh, Proc. Natl.  
25 Acad. Sci. U.S.A. **85**, 6272 (1988). Briefly, the spheroplasts were resuspended in 50 mM Tris-HCl pH 7.4, 5 mM EDTA and were lysed by vortex mixing with glass beads for three one-min periods at 4°C. Crude membranes were prepared from the lysates and binding assays with  
30  $^{125}\text{I}$ -CYP were performed by methods described previously. See H. Dohlman et al., Biochemistry **29**, 2335 (1990).

Figure 2 illustrates h $\beta$ AR ligand binding to membranes from pY $\beta$ AR2-transformed yeast cells. (A)  $B_{\text{max}}$  (maximum ligand bound) and  $K_d$  (ligand dissociation  
35 constant) values were determined by varying  $^{125}\text{I}$ -CYP concentrations (5 - 400 pM). Specific binding was

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defined as the amount of total binding (circles) minus nonspecific binding measured in the presence of 10  $\mu$ M (-) alprenolol (squares). A  $K_d$  of 93 pM for  $^{125}$ I-CYP binding was obtained and used to calculate agonist affinities (below). (B) Displacement of 18 pM  $^{125}$ I-CYP with various concentrations of agonists was used to determine apparent low affinity  $K_i$  values (non G protein coupled, determined in the presence of 50  $\mu$ M GTP) for receptor binding, squares; (-) isoproterenol, circles; (-) epinephrine, downward-pointing triangles; (+) isoproterenol, upward pointing triangles; (-) norepinephrine.

### COMPARATIVE EXAMPLE A

#### Ligand Binding Affinity for $h\beta$ AR Expressed in Yeast and Mammalian Cells

The binding data of Figures 2 (A) and (B) were analyzed by nonlinear least squares regression, see A. DeLean et al., Mol. Pharmacol. 21, (1982), and are presented in Table I. Values given are averages of measurements in triplicate, and are representative of 2 - 3 experiments. Binding affinities in yeast were nearly identical to those observed previously for  $h\beta$ AR expressed in mammalian cells.



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Table 1

Comparison of ligand Binding Parameters for High Level Expression of Human  $\beta$ -Adrenergic Receptor in Yeast and COS-7 Cells\*

5

	Yeast	Monkey	
	SC261 (pY $\beta$ AR2, pMTL9)	COS-7 (pBC12:h $\beta$ AR)	
-----			
125 <sub>I</sub> -CYP:			
10	<sup>1</sup> K <sub>d</sub>	0.093 nM $\pm$ 0.013	0.110 nM $\pm$ 0.009
	<sup>2</sup> B <sub>max</sub>	115 pmol/mg	24 pmol/mg
-----			
<sup>3</sup> K <sub>i</sub> (M):			
	(-) isoproterenol	103 $\pm$ 26	130 $\pm$ 15
15	(+) isoproterenol	3670 $\pm$ 420	4000 $\pm$ 184
	(-) epinephrine	664 $\pm$ 123	360 $\pm$ 30
	(-) norepinephrine	6000 $\pm$ 1383	5800 $\pm$ 373
-----			
20	*Values derived from Fig. 2 and H. Dohlman <u>et al.</u> , <u>Biochemistry</u> 29, 2335 (1990).; $\pm$ S.E.		
	<sup>1</sup> K <sub>d</sub> , ligand dissociation constant		
	<sup>2</sup> B <sub>max</sub> , maximum ligand bound		
	<sup>3</sup> K <sub>i</sub> , inhibition constant		

### EXAMPLE 3

#### 25      Agonist-Dependent Activation of Mating Signal Transduction in Yeast Expressing h $\beta$ AR

A second major function of a receptor is agonist-dependent regulation of downstream components in the signal transduction pathway. Because the pheromone-responsive effector in yeast is not known, indirect biological assays are the most useful indicators of receptor functionality. See K. Blumer and J. Thorner, Annu.Rev. Physiol. in press; I. Herskowitz, Microbiol. Rev. 52, 536 (1988). In yeast

cells expressing high concentrations of h $\beta$ AR, no agonist-dependent activation of the mating signal transduction pathway could be detected by any of the typical in vivo assays; for example, imposition of G1 arrest, induction of gene expression, alteration of morphology (so-called "shmoo" formation) or stimulation of mating. A likely explanation for the absence of responsiveness is that h $\beta$ AR was unable to couple with the endogenous yeast G protein.

#### EXAMPLE 4

##### Coexpression of h $\beta$ AR and Mammalian G<sub>s</sub> $\alpha$ -Subunit in Yeast

Expression of a mammalian G<sub>s</sub>  $\alpha$ -subunit can correct the growth defect in yeast cells lacking the corresponding endogenous protein encoded by the GPA1 gene. See C. Dietzel and J. Kurjan, Cell **50**, 1001 (1987). Moreover, specificity of receptor coupling in mammalian cells is conferred by the  $\alpha$ -subunit of G proteins. See L. Stryer and H. Bourne, Annu. Rev. Cell Biol. **2**, 391 (1988). Thus, coexpression of h $\beta$ AR and a mammalian G<sub>s</sub>  $\alpha$ -subunit (G<sub>s</sub> $\alpha$ ) in yeast was attempted to render the yeast responsive to adrenergic ligands. Accordingly, a cDNA encoding rat G<sub>s</sub> $\alpha$  under the control of the copper-inducible CUP1 promoter was introduced on a second plasmid, pYSK136Gas. See C. Dietzel and J. Kurjan, Cell **50**, 1001 (1987). In yeast (NNY19) coexpressing h $\beta$ AR and rat G<sub>s</sub> $\alpha$ , but containing wild-type GPA1, no adrenergic agonist-induced shmoo formation, a characteristic morphological change of yeast in response to mating pheromone, was observed.

#### EXAMPLE 5

##### Coexpression of h $\beta$ AR and Mammalian G<sub>s</sub> $\alpha$ -Subunit in Yeast Lacking an Endogenous G Prot in $\alpha$ -Subunit

To prevent interference by the endogenous yeast G protein  $\alpha$ -subunit, gpa1 mutant cells (strain

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8c) were used.

Yeast strain 8c (MATa ura3 leu2 his3 trp1  
gpa1::HIS3), I. Miyajima et al., Cell 50, 1011 (1987),  
carrying plasmids pYSK136G $\alpha$ s (TRP1), C. Dietzel and J.  
Kurjan, Cell 50, 1001 (1987), pMTL9 (LEU2), J. Salmeron  
et al., Mol. Cell. Biol. 9, 2950 (1989), and pY $\beta$ AR2  
(URA3) was maintained on glucose-free minimal selective  
plates containing 3% glycerol, 2% lactic acid, 50  $\mu$ M  
CuSO<sub>4</sub> and 3% galactose. Colonies were transferred to  
similar plates containing 0.5 mM ascorbic acid and the  
indicated adrenergic ligand(s). After 16-20 hours at  
30°C, the colonies were transferred to similar liquid  
media at a density of 10<sup>6</sup>-10<sup>7</sup> cells/ml and examined by  
phase contrast microscopy.

Morphologies of yeast cells cotransformed  
with pY $\beta$ AR2, pMTL9, and pYSK136G $\alpha$ s were examined after  
incubation with (A) no adrenergic agent; (B) 100  $\mu$ M (-)  
isoproterenol; (C) 100  $\mu$ M (-) isoproterenol and 50  $\mu$ M  
(-) alprenolol; and (D) 100  $\mu$ M (+) isoproterenol.  
Results showed that treatment of 8c cells coexpressing  
h $\beta$ AR and rat G $\alpha$  with the  $\beta$ -adrenergic agonist  
isoproterenol indeed induced shmoo formation, and that  
this effect was blocked by the specific antagonist  
alprenolol.

25

## EXAMPLE 6

### Coexpression of h $\beta$ AR and Mammalian G $\alpha$ -Subunit in Yeast Containing a $\beta$ -Galactosidase Signal Sequence

The isoproterenol-induced morphological  
response of 8c cells coexpressing h $\beta$ AR and rat G $\alpha$   
suggested that these components can couple to each  
other and to downstream components of the pheromone  
response pathway in yeast lacking the endogenous  
G  $\alpha$ -subunit. To confirm that the pheromone signaling  
pathway was activated by h $\beta$ AR and rat G $\alpha$ , agonist  
induction of the pheromone-responsive FUS1 gene  
promoter was measured in a strain of yeast derived from

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8c cells (8c1) in which a FUS1-lacZ gene fusion had been stably integrated into the genome. See S. Nomoto et al., EMBO J. 9, 691 (1990).

Strains 8c (Fig. 3, legend) and NNY19 (MATa  
5 ura3 leu2 his3 trp1 lys2 FUS1-LacZ::LEU2) were modified by integrative transformation with YIpFUS102 (LEU2), S. Nomoto et al., supra, and designated 8c1 and NNY19, respectively. These strains were transformed with pY $\beta$ AR2 and pYSK136G $\alpha$ s and maintained on minimal  
10 selective plates containing glucose and 50  $\mu$ M CuSO<sub>4</sub>. Colonies were inoculated into minimal selective media (3% glycerol, 2% lactic acid, 50  $\mu$ M CuSO<sub>4</sub>), grown to early log phase (OD<sub>600</sub> = 1.0), and induced for 12 hours by addition of 3% galactose. Cells were washed and  
15 resuspended in induction media (OD<sub>600</sub> = 5.0) containing 0.5 mM ascorbic acid and the indicated ligands. After a 4 hour incubation at 30°C, cells were harvested, resuspended into 1 ml of Z-buffer, see J. Miller, Experiments in Molecular Genetics (Cold Spring Harbor  
20 Laboratory, Cold Spring Harbor, NY, 1972), supplemented with 0.0075% SDS, and  $\beta$ -galactosidase activities were determined in 3 - 4 independent experiments as described previously. See J. Miller, supra.

Figure 3 shows a comparison of  $\beta$ -adrenergic  
25 agonist effects on pheromone-inducible gene activity.  $\alpha$ -MF, 10  $\mu$ M  $\alpha$ -mating factor; (-) ISO, 50  $\mu$ M (-) isoproterenol; (-) ALP, 50  $\mu$ M (-) alprenolol; (+) ISO, 100  $\mu$ M (+) isoproterenol. In 8c1 (gpa1) cells coexpressing h $\beta$ AR and rat G<sub>s</sub> $\alpha$ , a dramatic isoproterenol-  
30 stimulated induction of  $\beta$ -galactosidase activity was observed. Agonist stimulation was stereoselective and was blocked by addition of a specific antagonist. Agonist responsiveness was dependent on expression of both h $\beta$ AR and rat G<sub>s</sub> $\alpha$ , and required a strain in which  
35 the endogenous G protein  $\alpha$ -subunit was disrupted. The final  $\beta$ -galactosidase activity achieved in response to isoproterenol in transformed 8c1 cells was comparable

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to that induced by  $\alpha$ -factor in nontransformed cells that express GPA1 (NNY19), although basal  $\beta$ -galactosidase activity in NNY19 cells was considerably lower than in 8c1 cells. Taken together, 5 our results indicated that coexpression of h $\beta$ AR and rat  $G_s\alpha$  was sufficient to place under catecholamine control key aspects of the mating signal transduction pathway in yeast. However, the adrenergic agonist did not stimulate mating in either 8c cells or NNY19 cells 10 coexpressing h $\beta$ AR and rat  $G_s\alpha$ , in agreement with recent observations that yeast pheromone receptors, in addition to binding pheromones, participate in other recognition events required for mating. See A. Bender and G. Sprague, Genetics 121, 463 (1989).

15 h $\beta$ AR stimulates adenylate cyclase in animal cells via the action of the  $\alpha$ -subunit of its G protein. In contrast, mating factor receptors in yeast trigger their effector via the action of the  $\beta\gamma$  subunits. M. Whiteway et al., Cell 56, 476 (1989). Our present 20 results indicate that activation of h $\beta$ AR in yeast leads to dissociation of mammalian  $G_s\alpha$  from yeast  $\beta\gamma$ , and it is the  $\beta\gamma$  subunits that presumably elicit the response.

The foregoing examples are illustrative of the present invention, and are not to be construed as 25 limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

-20-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: King, Klim  
Dohlman, Henrik G.  
Caron, Mark G.  
Lefkowitz, Robert J.
- (ii) TITLE OF INVENTION: Expression of G Protein Coupled  
Receptors in Yeast
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Kenneth D. Sibley; Bell, Seltzer, Park and  
Gibson
  - (B) STREET: Post Office Drawer 34009
  - (C) CITY: Charlotte
  - (D) STATE: North Carolina
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 28234
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: PCT/US91/06605
  - (B) FILING DATE: 12-SEP-1991
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 07/581714
  - (B) FILING DATE: 13-SEP-1990
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Sibley, Kenneth D.
  - (B) REGISTRATION NUMBER: 31,665
  - (C) REFERENCE/DOCKET NUMBER: 5405-17-1
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 919-881-3140
  - (B) TELEFAX: 919-881-3175
  - (C) TELEX: 575102

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 80 base pairs

SUBSTITUTE SHEET

-21-

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 30..80

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCAACG	TTGGATCCAA	GAATCAAAA	ATG	TCT	GAT	GCG	GCT	CCT	TCA	TTG	53
			Met	Ser	Asp	Ala	Ala	Pro	Ser	Leu	
			1				5				

AGC	AAT	CTA	TTT	TAT	GAC	GTC	ACG	CAG			80
Ser	Asn	Leu	Phe	Tyr	Asp	Val	Thr	Gln			
	10					15					

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Ser	Asp	Ala	Ala	Pro	Ser	Leu	Ser	Asn	Leu	Phe	Tyr	Asp	Val	Thr
1				5					10					15	
															Gln

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AACGTT

6

## THAT WHICH IS CLAIMED IS:

1. A transformed yeast cell containing a first heterologous DNA sequence which codes for a mammalian G protein coupled receptor and a second heterologous DNA sequence which codes for a mammalian G protein  $\alpha$  subunit (mammalian  $G_\alpha$ ), wherein said first and second heterologous DNA sequences are capable of expression in said cell, and wherein said cell is incapable of expressing an endogenous G protein  $\alpha$ -subunit (yeast  $G_\alpha$ ).

2. A transformed yeast cell according to claim 1, wherein said first heterologous DNA sequence is carried by a plasmid.

3. A transformed yeast cell according to claim 1, wherein said second heterologous DNA sequence is carried by a plasmid.

4. A transformed yeast cell according to claim 1, wherein said mammalian G protein  $\alpha$  subunit is selected from the group consisting of  $G_s$   $\alpha$  subunits,  $G_i$   $\alpha$  subunits,  $G_o$   $\alpha$  subunits,  $G_z$   $\alpha$  subunits, and transducin  $\alpha$  subunits.

5. A transformed yeast cell according to claim 1 which expresses a complex of the G protein  $\beta$  subunit and the G protein  $\gamma$  subunit ( $G_{\beta\gamma}$ ).

6. A transformed yeast cell according to claim 5 which expresses endogenous  $G_{\beta\gamma}$ .



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7. A transformed yeast cell according to claim 1, wherein said first heterologous DNA sequence codes for a mammalian G protein-coupled receptor selected from the group consisting of dopamine  
5 receptors, muscarinic cholinergic receptors,  $\alpha$ -adrenergic receptors,  $\beta$ -adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors.

8. A transformed yeast cell according to claim 1 further comprising a third heterologous DNA sequence, wherein said third heterologous DNA sequence comprises a pheromone-responsive promotor and an  
5 indicator gene positioned downstream from said pheromone-responsive promoter and operatively associated therewith.

9. A transformed yeast cell according to claim 8, wherein said pheromone responsive promoter is selected from the group consisting of the BAR1 gene promoter and the FUS1 gene promoter, and wherein said  
5 indicator gene is selected from the group consisting of the HIS3 gene and the LacZ gene.

heterologous DNA sequence which codes for a mammalian  $G_{\alpha}$ , wherein said first and second heterologous DNA sequences are capable of expression in said cell,  
10 wherein said cell is incapable of expressing endogenous  $G_{\alpha}$ , and wherein said cell expresses  $G_{\beta\gamma}$ ;  
contacting said compound to said cell; and  
detecting the rate of dissociation of  $G_{\alpha}$  from  $G_{\beta\gamma}$  in said cell.

11. A method according to claim 10, wherein said yeast cells are provided in an aqueous solution and said contacting step is carried out by adding said compound to said aqueous solution.

12. A method according to claim 10, wherein said mammalian G protein  $\alpha$  subunit is selected from the group consisting of  $G_s$   $\alpha$  subunits,  $G_i$   $\alpha$  subunits,  $G_o$   $\alpha$  subunits,  $G_z$   $\alpha$  subunits, and transducin  $\alpha$  subunits.

13. A method according to claim 10, wherein said yeast cell expresses endogenous  $G_{\beta\gamma}$ .

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14. A method according to claim 10, wherein said first heterologous DNA sequence codes for a mammalian G protein-coupled receptor selected from the group consisting of dopamine receptors, muscarinic cholinergic receptors,  $\alpha$ -adrenergic receptors,  $\beta$ -adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors.

15. A method according to claim 10, said yeast cell further comprising a third heterologous DNA sequence, wherein said third heterologous DNA sequence comprises a pheromone-responsive promotor and an indicator gene positioned downstream from said pheromone-responsive promoter and operatively associated therewith;

and wherein said detecting step is carried out by monitoring the expression of said indicator gene in said cell.

16. A DNA expression vector capable of expressing a transmembrane protein into the cell membrane of yeast cells, comprising:

a first segment comprising at least a fragment of the extreme amino-terminal coding sequence of a yeast G protein coupled receptor; and

a second segment downstream from said first segment and in correct reading frame therewith, said second segment comprising a DNA sequence encoding a heterologous G protein coupled receptor.

17. A DNA expression vector according to claim 16, wherein a fragment of the extreme amino-terminal coding sequence of said heterologous G protein coupled receptor is absent.

18. A DNA expression vector according to claim 16, wherein said first and second segments are operatively associated with a promoter operative in a yeast cell.

19. A DNA expression vector according to claim 18, wherein said promoter is the GAL1 promoter.

20. A DNA expression vector according to claim 16, wherein said first segment comprises at least a fragment of the extreme amino-terminal coding sequence of a yeast pheromone receptor.

21. A DNA expression vector according to claim 16, wherein said first segment comprises at least a fragment of the extreme amino-terminal coding sequence of a yeast pheromone receptor selected from the group consisting of the STE2 gene and the STE3 gene.

22. A DNA expression vector according to claim 16, further comprising at least a fragment of the 5'-untranslated region of a yeast G protein coupled receptor gene positioned upstream from said first segment and operatively associated therewith.

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23. A DNA expression vector according to claim 16, further comprising at least a fragment of the 5'-untranslated region of a yeast pheromone receptor gene positioned upstream from said first segment and operatively associated therewith.

24. A DNA expression vector according to claim 23, wherein said yeast pheromone receptor gene is selected from the group consisting of the STE2 gene and the STE3 gene.

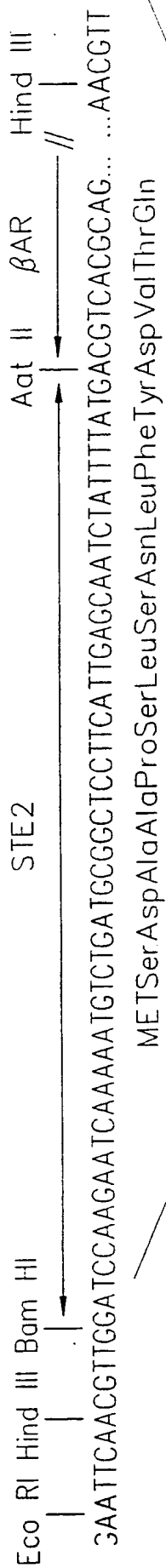
25. A DNA expression vector according to claim 16, said vector comprising a plasmid.

26. A DNA expression vector according to claim 16, said second segment comprising a DNA sequence encoding a mammalian G protein coupled receptor.

27. A DNA expression vector according to claim 16, said second segment comprising a DNA sequence encoding a mammalian G protein-coupled receptor selected from the group consisting of dopamine receptors, muscarinic cholinergic receptors,  $\alpha$ -adrenergic receptors,  $\beta$ -adrenergic receptors, opiate receptors, cannabinoid receptors, and serotonin receptors.

28. A yeast cell carrying a DNA expression vector according to claim 16.

FIG. 1A.



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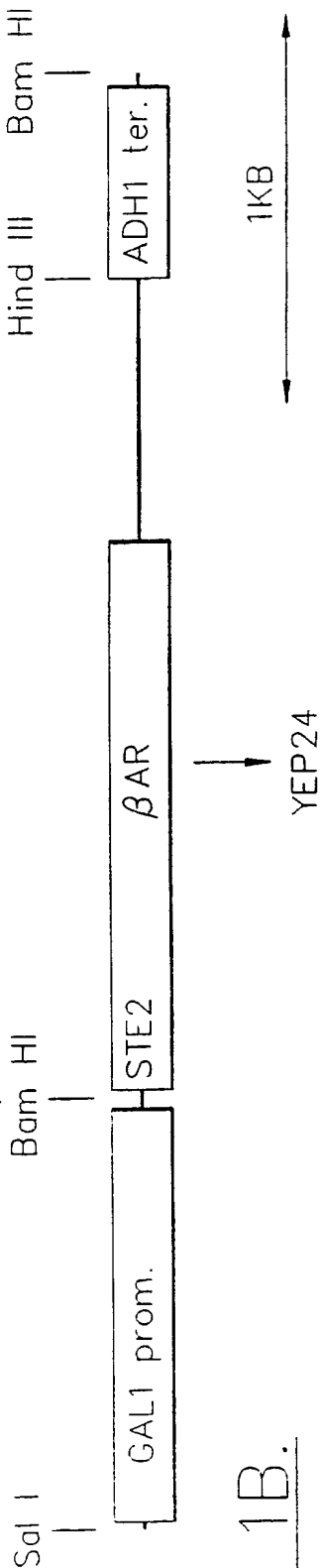
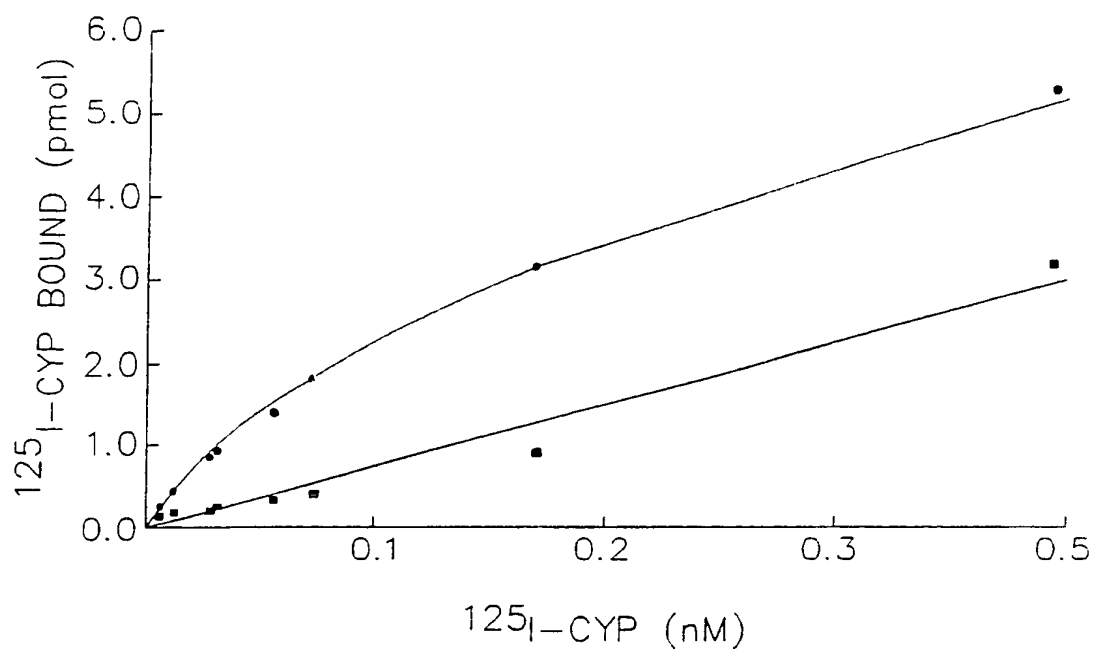
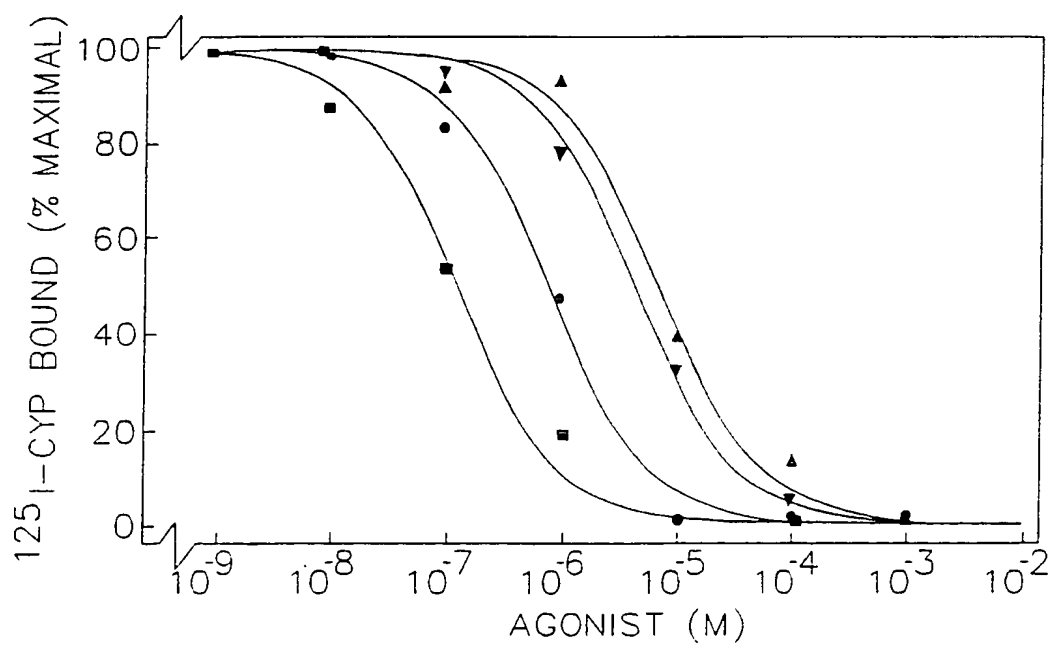
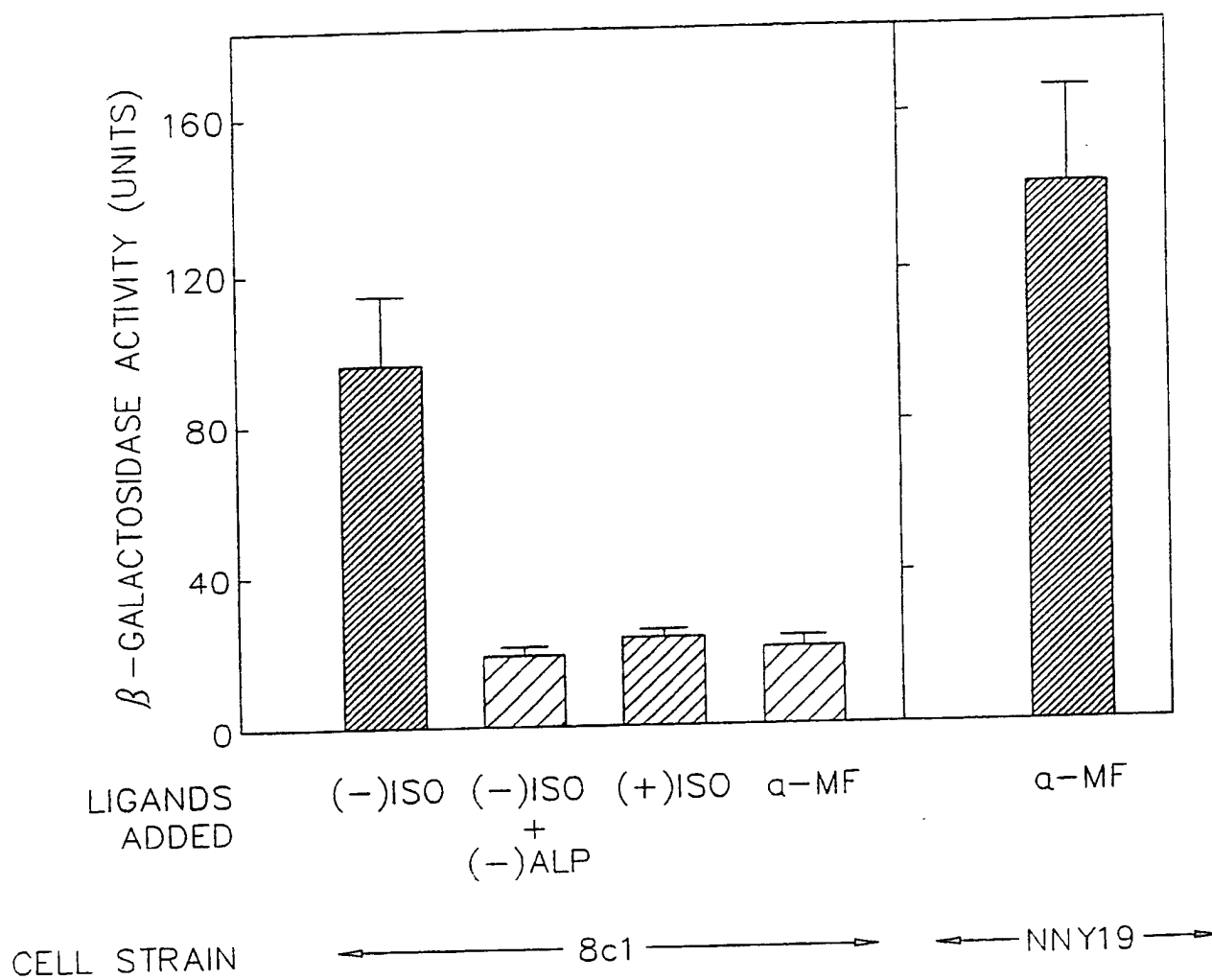


FIG. 1B.

FIG. 2A.FIG. 2B.

FIG. 3.



# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US91/06605**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12N 1/20; C12! 1/00; GOIN 33/48, 33/566 USCL.: 435/4,252.3; 436/63,501											
<b>II. FIELDS SEARCHED</b> <div style="text-align: right; margin-right: 100px;">Minimum Documentation Searched <sup>7</sup></div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%;">Classification System</th> <th style="width: 75%;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px;">US.CL.</td> <td style="padding: 5px;">435/4, 252.3; 436/63,501</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	US.CL.	435/4, 252.3; 436/63,501					
Classification System	Classification Symbols										
US.CL.	435/4, 252.3; 436/63,501										
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%;">Category <sup>*</sup></th> <th style="width: 70%;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 10px;">Y</td> <td style="padding: 10px;">Annual Review of Cell Biology, Volume 2, issued 1986. Stryer et al., "G Proteins: A Family of Singnal Transducers," pages 391-419. see entire document.</td> <td style="text-align: center; vertical-align: top; padding: 10px;">1-28</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 10px;">Y</td> <td style="padding: 10px;">WO.A. 90/05780 (Civelli et al.) 31 May 1990. see column 29. line 26-column 30. line 8.</td> <td style="text-align: center; vertical-align: top; padding: 10px;">1-28</td> </tr> </table>			Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	Y	Annual Review of Cell Biology, Volume 2, issued 1986. Stryer et al., "G Proteins: A Family of Singnal Transducers," pages 391-419. see entire document.	1-28	Y	WO.A. 90/05780 (Civelli et al.) 31 May 1990. see column 29. line 26-column 30. line 8.	1-28
Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>									
Y	Annual Review of Cell Biology, Volume 2, issued 1986. Stryer et al., "G Proteins: A Family of Singnal Transducers," pages 391-419. see entire document.	1-28									
Y	WO.A. 90/05780 (Civelli et al.) 31 May 1990. see column 29. line 26-column 30. line 8.	1-28									
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>											
<b>IV. CERTIFICATION</b> <table style="width: 100%;"> <tr> <td style="width: 50%; vertical-align: top;">         Date of the Actual Completion of the International Search   <b>01 November 1991</b>          International Searching Authority   <div style="text-align: center;">ISA/US</div> </td> <td style="width: 50%; vertical-align: top;">         Date of Mailing of this International Search Report  <div style="text-align: center; font-size: 1.2em;">18 DEC 1991</div>         Signature of Authorized Officer  <div style="text-align: center;">Lori Yuan</div> </td> </tr> </table>			Date of the Actual Completion of the International Search  <b>01 November 1991</b> International Searching Authority  <div style="text-align: center;">ISA/US</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em;">18 DEC 1991</div> Signature of Authorized Officer <div style="text-align: center;">Lori Yuan</div>							
Date of the Actual Completion of the International Search  <b>01 November 1991</b> International Searching Authority  <div style="text-align: center;">ISA/US</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em;">18 DEC 1991</div> Signature of Authorized Officer <div style="text-align: center;">Lori Yuan</div>										

